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**Impacts of pH and temperature on soil bacterial 3-hydroxy fatty acids:
development of novel terrestrial proxies**

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Abstract: Gram-negative bacterial 3-hydroxy fatty acids (3-OH-FAs) biomarkers are widespread in a variety of environments including both marine and terrestrial sediments (including speleothems). In this study we analysed the hydroxylated membrane lipids of 26 soil samples from an altitudinal transect of Shennongjia Mountain (Mt.) in central China to study the environmental factors controlling the relative distribution of 3-OH-FAs. Our results show that both the ratio of the summed *iso* and *anteiso* to the total amount of *normal* 3-OH-FAs (RIAN), and the ratio of summed *iso* and *anteiso* to the total amount of all 3-OH-FAs (Branched Index) were primarily related to the pH of soil ($R^2 = 0.70$ and 0.70 , respectively). Additionally, the *anteiso* to *normal* 3-hydroxy fatty acids ratio of the C_{15} and C_{17} homologues (RAN₁₅ and RAN₁₇) shows a significant negative correlation with mean annual air temperature (MAAT) ($R^2=0.51$ and 0.48 , respectively). When comparing the 3-OH-FA based indices with established glycerol dialkyl glycerol tetraether (GDGT) based indices from the same soil samples, the RIAN and Branched Index show strong linear correlations with the cyclisation ratio of branched tetraethers (CBT) ($R^2 = 0.77$ and 0.74 , respectively), and the RAN₁₅ and RAN₁₇ show negative correlations with the MBT/CBT-MAAT (MBT, methylation index of branched tetraethers) ($R^2 = 0.61$ and 0.36 , respectively). Our new field-based correlations demonstrate the physiological response of Gram-negative bacterial cell membranes to the external environment and suggest that 3-hydroxy fatty

acids can be applied in palaeoenvironmental studies to estimate past MAAT and soil pH.

Keywords: proxy, 3-hydroxy fatty acid, soil, temperature, soil pH, palaeoclimate

1. Introduction

A wide range of environmental information from both terrestrial and marine realms is required from palaeoclimate archives to better understand the climate system and to provide a palaeoclimatic context for predictions of future rates of climate change, impact and Earth System sensitivity. To date, various geochemical proxies based on inorganic and organic fossil remains have been applied in order to reconstruct past environmental parameters. Organic biomarkers have become widely deployed tools in the reconstruction of past environmental conditions, due in part to: a) the sensitive physiological responses of cell membranes and structural lipids to the external environment and b) their relatively high preservation potential (Summons, 1993; Eglinton and Eglinton, 2008). Since the 1960's a large array of lipid biomarkers with applications in palaeoclimatology have been identified, including plant waxes, hopanes, alkanes and glycerol dialkyl glycerol tetraethers (GDGTs). Two proxies, U_{37}^K (Brassell et al., 1986; Prahl and Wakeham, 1987; Sachs et al.,

2001; Haug et al., 2005) and TEX₈₆ (Schouten et al., 2002; Kim et al., 2008), based on C₃₇ alkenones and GDGTs, respectively, have been widely employed to calculate sea surface temperatures (SST) as far back as the Jurassic (Jenkyns et al., 2012).

Numerous lipid biomarkers derived from terrestrial organic matter are preserved in lacustrine (e.g. Castañeda and Schouten, 2011) and marine (Pancost and Boot, 2004) archives. Commonly utilised biomarker groups include higher plant derived *n*-alkyl compounds, terpenoids and lignins (Pancost and Boot, 2004) and soil bacterial branched-GDGTs (Weijers et al., 2007a). Such compounds can be used to reconstruct general changes in inputs and provenance of terrestrial material (Pancost and Boot, 2004, Seki et al., 2014). Compound specific isotopic analyses, particularly on higher plant waxes have expanded the range of palaeoclimatic applications, for example, D/H analysis is used to infer changes in past hydrological regimes (Sachse et al., 2012 and reference therein) and the $\delta^{13}\text{C}$ analysis of higher plant biomarkers is a powerful tool to constrain changes in C₃ vs C₄ vegetation (e.g. Hughen et al., 2004). More recently, the bacterial GDGT based cyclization of branched tetraether (CBT) proxy has been developed and applied for the reconstruction of soil pH in terrestrial settings (Weijers et al., 2007b). In parallel, the combination of CBT with the methylation of branched tetraethers (MBT) index may be deployed to estimate past variations in mean annual air temperature (MAAT) (Weijers et al., 2007b). However, overall, relatively less attention has

84 been paid to terrestrial environments, compared to the marine realm, due to
85 the historical paucity of ubiquitous biomarkers with quantitative
86 palaeoclimatic utility. Thus the discovery and development of new quantitative
87 terrestrial proxies is of major significance. Targets of particular value are
88 compounds preserved in both aquatic and terrestrial sediments, as this
89 facilitates the correlation and comparison of palaeoclimatic records between
90 marine and terrestrial environments (Pancost and Boot, 2004; Castañeda and
91 Schouten, 2011).

92 Lipopolysaccharide (LPS) is the main component of the outer membrane of
93 Gram-negative bacteria. Lipid A, a constituent part of LPS, consists of
94 glucosamine units and fatty acids, many of the latter are 3-hydroxy fatty acids
95 (3-OH-FAs), also known as ω -hydroxy fatty acids, with carbon numbers from
96 C₁₀ to C₁₈ (Fig. 1) (Wollenweber and Rietschel, 1990; Szponar et al., 2002;
97 Szponar et al., 2003). These are bound to the glucosamine unit either by ester
98 bonds or amide bonds (Wollenweber et al., 1982; Kumar et al., 2002). A
99 significant body of literature demonstrates that the dominant precursors for
100 C₁₀-C₁₈ 3-OH-FAs compounds in the environment are Gram-negative bacteria
101 (Wollenweber and Rietschel, 1990; Saraf et al., 1997; Szponar et al., 2002;
102 Keinänen et al., 2003; Szponar et al., 2003). Such that 3-OH-FAs in the C₁₀-C₁₈
103 range are accepted as diagnostic markers for the characterisation and
104 quantification of Gram-negative bacterial LPS (i.e. endotoxins) in clinical and
105 environmental studies (Sonesson et al., 1990; Mielniczuk et al., 1993; Saraf et

al., 1997; Szponar et al., 2002; Keinänen et al., 2003; Wakeham et al., 2003; Lee et al., 2004; Ferrando et al., 2005; Kračnik et al., 2006; Lee et al., 2007). However, one study suggests C₁₀-C₁₈ 3-OH-FAs are also produced by Gram-positive *Lactobacillus plantarum* (Sjogren et al., 2003). Additionally, long chain 3-OH-FAs (C₂₆-C₃₀) are reportedly derived from microalgae of the class Eustigmatophyceae (Volkman et al., 1998).

3-OH-FAs with carbon chain lengths from C₁₀ to C₁₈ have been used to quantify and characterize the Gram-negative bacterial community in samples from a diverse array of environments, including atmospheric aerosols (Lee et al., 2004) and marine dissolved organic matter (DOM) (Wakeham et al., 2003). However, thus far, the relationship between 3-OH-FAs and environmental parameters has not been systematically investigated in soils or sediments with the aim of exploring the possible utility of these ubiquitous fatty acids as quantitative environmental proxies.

We explore the distribution of these microbial biomarkers on Mt. Shennongjia, a national reserve located at the northwest of Hubei province, central China (31°15'-31°57'N, 109°59'-110°58'E) (Fig. 2), to test whether 3-OH-FAs record a signal of sensitive and differential physiological responses, by Gram-negative bacteria, to ambient environmental conditions, and if novel quantitative proxies could be independently established for palaeoenvironmental reconstruction.

2. Methods

2.1 Sampling site

Mt. Shennongjia, with an altitude of 3105 m above sea level (m.a.s.l.), is located in a climatic region dominated by the Asian monsoon. Five meteorological stations established at different altitudes in this region provide a precise altitudinal record of meteorological conditions. Moreover, a large gradient of soil pH, MAAT and mean annual precipitation (MAP) prevails on Mt. Shennongjia, making it a natural laboratory to test the relationship between 3-OH-FAs and environmental parameters. Average climatic conditions trend from warm and dry conditions at the base (315 m.a.s.l.) to cool and wet conditions at the highest sampling site (2840 m.a.s.l.), with MAAT varying from 1.9 °C to 14.7 °C; MAP from 1226mm to 3313mm and soil humidity from 11.6% to 55.6% (Supplementary data Table 1). Soil pH varies from 4.49 to 7.98, however it has no causal relationship with altitude, MAAT, MAP or soil humidity (Fig. 3), indicating the pH is an independent environmental factor, likely controlled by changes in bedrock geology. Both MAAT ($R^2=0.995$) and MAP ($R^2= 0.951$) are highly correlated to altitude (and thus co-vary), according to the linear regressions between altitude and climatic factors reported by Li and Manfred (2002) based on the climatic data from the local meteorological station (Songpei, 930 m.a.s.l.) and the four subsidiary stations in the Mt. Shennongjia area (Yangriwan, 460 m.a.s.l.; Dajiuhu, 1700 m.a.s.l.; Changyanwu, 2300 m.a.s.l.; the mountain observation tower, 2930 m.a.s.l.).

The vertical vegetation distribution on Shennongjia Mountain is very distinct. Based on the latest investigation by Zhao et al., (2005), the vegetation zones along the elevation gradient were described as follows: evergreen broadleaved forest zone at altitudes below 900 m.a.s.l.; mixed evergreen and deciduous broadleaved forest between 900 and 1500 m.a.s.l.; deciduous broadleaved forest zone between 1500 and 2000 m.a.s.l.; mixed conifer and deciduous broadleaved forest between 2000 and 2400 m.a.s.l.; and sub-alpine conifer forest zone (including sub-alpine shrubs and meadows) above altitudes of 2400 m.a.s.l. (Zhao et al., 2005).

2.2 Sample collection

Twenty-six soil samples were collected along an altitude transect of Mt. Shennongjia between 315 and 2840 m.a.s.l. at altitudinal intervals of ca. 200 m. The topmost leaf-litter layer was removed before sampling. Samples from each soil are derived from the depth intervals between 0 to 10 cm. The samples were wrapped in pre-combusted aluminium foil and then stored with ice bags. Upon arrival at the laboratory, the soils were stored at -20°C in a freezer before freeze drying. The location of sampling sites was measured by a portable GPS instrument (Supplementary data Table 1). Soil moisture was determined by measuring the weight difference before and after freeze drying. Then the dry samples were ground into powder with a pestle and mortar. A late Holocene lake sediment sample was taken from a core collected from Tianchi Lake in

Gansu Province, China (Zhou et al., 2010) (Fig. 2). A stalagmite sub-sample was obtained from the HS4 stalagmite which was collected from Heshang Cave, Hubei province, China (Hu et al., 2008) (Fig. 2). A marine sediment sample was collected from IODP Site M0060, in the Baltic Sea.

2.3 Soil pH measurement

Soil pH data either comes from or was measured following the method of Yang et al. (2015). Soil samples were mixed with ultrapure water in a ratio of 1:2.5 (g/mL). After standing for 30 min, the supernatant pH was measured, using a meter with a precision of ± 0.01 . The pH was measured three times and the mean value was taken as the final pH.

2.4 Extraction and clean-up methods

The soil, stalagmite and marine sediment samples were subjected to acid hydrolysis following an optimized acid digestion method (Wang et al., 2012). 10g of homogenized sample was mixed with 30 mL pre-cleaned HCl (3M), and then refluxed under 130 °C for 3h. After cooling, the solution was extracted x3 with DCM, to yield the Total Lipid Extract (TLE). The lake sediment was hydrolysed by 0.3M KOH methanolic solution containing 5% water, heating under 70 °C for 2h in a closed test tube. The neutral fraction was extracted with *n*-hexane:DCM (9:1, v/v) and then the acid fraction was extracted with DCM after adjusting the pH of the residues below 2 with pre-cleaned HCl. The TLE (soils, stalagmite and marine sediment) and acid fraction (lake sediment)

was methylated by $\text{BF}_3\text{-MeOH}$ solution at 70 °C for 1.5h. The resulting fatty acid methyl esters (FAMEs) were separated into non-OH-FAMEs and OH-FAMEs following the method described by Jenske and Vetter (2008). Non-OH-FAMEs were eluted in the first fraction with a solvent mixture of n-hexane and ethyl acetate (v/v =98:2), whereas OH-FAMEs were obtained by elution with 100% ethyl acetate. The OH-FAME fraction was further derivatised by BSTFA (N, O-bis (trimethylsilyl) trifluoroacetamide) at 70 °C for 1.5h before further analysis by gas chromatogram-mass spectrometer (GC-MS).

2.5 Instrumentation

The 3-OH-FAs from soils, stalagmite and marine sediment were analysed by an Agilent 7890A gas chromatogram and 5975C mass spectrometer (GC-MS) equipped with a ZB-5MS fused silica capillary column (60 m \times 0.25 mm \times 0.25 μm) at the China University of Geosciences (Wuhan). The GC oven temperature was ramped from 70 °C to 200 °C at 10 °C/min, then to 310 °C at 3 °C/min, held at 310 °C for 47 min. The carrier gas was Helium (99.999%) and the gas flow was 1.0 mL/min. The 3-OH-FAs from Tianchi Lake were analysed by a 7890B gas chromatogram and 5977A mass spectrometer equipped with a BP5MS fused silica capillary column (60 m \times 0.32 mm \times 0.25 μm) at the University of Birmingham. The ionization energy of the mass spectrometer was set at 70 eV. The 3-OH-FAs were identified based on their mass spectra and relative retention times (Fig. 4). All the 3-OH-FAs TMSi esters show diagnostic

fragment ions, m/z 175 ($[\text{CH}_3]_3\text{SiO} = \text{CHCH}_2\text{CO}_2\text{CH}_3$), due to the cleavage between C_3 and C_4 , and M-15 (base peak) results from a loss of a CH_3 group. Other characteristic ions include m/z 103, 89, 133, 159, and M^+-31 (Eglinton et al., 1968; Mielniczuk et al., 1993; Volkman et al., 1999). Samples were analysed in duplicate or triplicate to obtain the analytical errors of the proxies. The analytical errors are graphically illustrated in the relevant figures with error bars.

3. Results and discussion:

3.1 Distribution of 3-OH-FAs

A total of 26 soil samples from Mt. Shennongjia were analysed. The carbon number of the 3-OH-FAs ranges from C_{10} to C_{18} , including *iso*- C_{11} , C_{13} , C_{15} , C_{16} , C_{17} and *anteiso*- C_{13} , C_{15} , C_{17} 3-OH-FAs. *n*- C_{14} is the dominant homologue (Fig. 5). The distribution of the Mt. Shennongjia 3-OH-FAs is akin to that derived from the LPS component of the outer bacterial membrane of Gram-negative bacteria (Klok et al., 1988). Thus we assume that the 3-OH-FAs measured in the Mt. Shennongjia soils originate from the soil dwelling consortia of Gram-negative bacteria. Furthermore, the suite of 3-OH-FAs compounds detected is similar to that reported from stalagmites (Blyth et al., 2006; Huang et al., 2008; Wang et al., 2012), marine DOM (Wakeham et al., 2003) and lake sediments (Matsuda and Koyama, 1977; Zhang et al., 2014), although the dominant homologue varies between C_{12} , C_{14} to C_{16} in these different sample types, and

the relative abundance of each individual compound fluctuates from sample to sample.

3.2 pH impact on 3-OH-FAs and potential proxies

Organic geochemical method development work on acid digestion of speleothem and cave samples from Heshang cave, located ca. 120 km from Mt. Shennongjia in central China (Wang et al., 2012; Huang et al., 2008), revealed that a suite of 3-OH-FAs were readily extractable and relatively abundant compared to established palaeoclimate biomarkers (e.g. plant waxes). This prompted an investigation of the distributions of these compounds along the Mt. Shennongjia altitudinal gradient and the current study of their empirical relationship to environmental parameters. Below we discuss in more detail the most promising 3-OH-FA indices we have identified. In Table 3 in the Supplementary data we include a list of all the 3-OH-FA based indices we tested, including those which showed low or insignificant correlations with environmental parameters (MAAT, soil pH, MAP, soil moisture and altitude).

The first group of indices we discuss are those which show relatively high correlations with soil pH. Recent work has demonstrated that pH is a key environmental parameter in controlling soil bacterial community structure and diversity (Bååth and Anderson, 2003; Lauber et al., 2009; Griffiths et al., 2011; Shen et al., 2013; Zhang et al., 2015). In particular, Giotis et al. (2007) found that a strain of Gram-negative bacterium increased/decreased the proportion of branched-chain fatty acids in higher pH/lower pH conditions. Our results from

the Mt. Shennongjia transect show that the ratio of the total sum of *iso* and *anteiso* 3-OH-FAs to the total amount of *normal* 3-OH-FAs i.e., the Branching Ratio (equation 1), has a positive correlation with the pH value of soils (Fig. 6a). The Branching Ratio is defined as follows:

$$\text{Branching Ratio} = (I + A)/N \quad (1)$$

Where I represents the sum of all the *iso* 3-OH-FAs, A represents the sum of all the *anteiso* 3-OH-FAs, and N represents the sum of all the *normal* 3-OH-FAs.

When plotting the Branching Ratio against the pH value of the soils, there is an exponential relationship between the two ($R^2 = 0.76$), with the Branching Ratio increasing significantly from 0.31 at pH 4.49 to 0.61 at pH 7.98 (Fig. 6a). Notably, the Branching Ratio shows no obvious correlation with MAAT, MAP or soil humidity (Fig. 7a-c, Supplementary data Table 3).

The fact that pH on Mt. Shennongjia does not correlate with other measured parameters (MAAT, MAP, soil humidity) precludes problems of covariance and gives us confidence that the Branching Ratio does primarily record a signal of environmental pH.

Equation (1) and Figure 6a clearly indicate proportionally less branched 3-OH-FAs, including *iso* and *anteiso* isomers, when pH decreases, and thus a lower pH yields a lower Branching Ratio value. This is consistent with the general observation that bacteria can alter the branching and cyclicity of their fatty acid membrane lipids in response to ambient environmental factors

(Denich et al., 2003). Branching in fatty acids increases the fluidity (Russell and Fukunaga, 1990) and permeability (McElhaney et al., 1973) of the cytoplasmic membrane.

We suggest that the observation of a decreasing Branching Ratio at lower pH reflects chemiosmotic coupling, i.e. the production of fewer branched homologues, producing a less fluid / more impermeable membrane to counteract steeper proton gradients. The existence and maintenance of a proton gradient over bacterial cell membranes is vital for the energy supply of a cell (Mitchell, 1966) and involves the trapping of proton conducting water molecules in the lipid core of the membranes (Nagle and Morowitz, 1978; Wikström et al., 2015). The high significance of the exponential regression supports this hypothesis. The proton gradient over the bacterial cell membranes will be largely determined by ambient proton concentrations and pH is a nonlinear function, being the negative logarithm of ambient proton concentrations. Given the exponential relationship between pH and the Branching Ratio (Fig. 6a) and the definition of pH as the negative logarithm of the proton concentration, it is possible to obtain a linear relationship between the two by defining an alternative index:

$$\text{RIAN} = -\log(\text{Branching Ratio}) \quad (2)$$

When plotting the ratio of the total sum of *iso* and *anteiso* 3-OH-FAs to the total amount of *normal* 3-OH-FAs (RIAN) against the pH of the soils resulted in the following linear correlation (Fig. 6b):

$$\text{RIAN} = 1.11 - 0.10 \times \text{pH} \quad (R^2 = 0.70, p < 0.001) \quad (3)$$

Thus we propose the following novel pH proxy for application to terrestrial palaeoclimatic archives:

$$\text{pH} = 11.10 - 10.00 \times \text{RIAN} \quad (R^2 = 0.70, p < 0.001, \text{RMSE} = 0.54) \quad (4)$$

In addition to Branching Ratio and RIAN, we find that the ratio of summed branched homologues to the sum of all 3-OH-FA homologues (Branched Index) and the ratio of summed *iso* to summed *normal* 3-OH-FA homologues (RIN) also show strong correlations with soil pH ($R^2 = 0.70$ and $R^2 = 0.67$, respectively) (Fig. 6c, d, Supplementary data Table 3). The equations for the Branched Index and RIN are:

$$\text{Branched Index} = (I + A) / (I + A + N) \quad (5)$$

$$\text{RIN} = I / N \quad (6)$$

Where I represents the sum of all the *iso* 3-OH-FAs, A represents the sum of all the *anteiso* 3-OH-FAs, and N represents the sum of all the *normal* 3-OH-FAs. The possible advantages of these alternative indices are that the Branched Index is bounded at values between 0 and 1 (the Branching ratio and the RIAN are unbounded), whereas RIN only utilises the *normal* and *iso* homologues and does not require measurement of the *anteiso* homologues. RIN may prove to have a practical advantage as the *anteiso* homologues occur in the lowest abundance in our samples (see Figure 5) and may be hard to accurately integrate in some environmental samples where the overall abundance or preservation of 3-OH-FAs is lower.

All the ratios and indices presented show positive or negative correlations (R²= 0.67 to 0.76, p<0.001) with pH (Fig. 6) but show no obvious correlation with MAAT, MAP or soil humidity (Fig. 7 and Supplementary data Table 3). All the ratios and indices appear to be independent measures of the decreased/increased degree of branching of 3-OH-FAs with lower/higher pH. As discussed above, for the Branching Ratio, this suggests a causal relationship with soil pH which we argue reflects chemiosmotic coupling, i.e. the production of fewer or more branched homologues to control membrane fluidity/permeability in response to proton gradients across bacterial cell membranes. This is comparable with the suggestion of Weijers et al. (2007b) that a lower/higher degree of methylation of branched GDGTs in lower/higher pH conditions reflects chemiosmotic coupling and is consistent with the finding of Bardy et al. (2009) that the contribution of branched C₁₅ and C₁₇ alkanolic acids relative to their linear homologues decreased with pH in a podzolic sequence in the Amazon basin.

Based on the linear correlations showed in Fig. 6c, d, we obtain the following equations with pH for the Branched Index and RIN:

$$\text{Branched Index} = -0.03 + 0.05 \times \text{pH} \quad (7)$$

$$\text{RIN} = -0.21 + 0.08 \times \text{pH} \quad (8)$$

Thus we propose the additional novel pH proxies for application to terrestrial palaeoclimatic archives:

$$\text{pH} = 0.60 + 20.00 \times \text{Branched Index} (R^2 = 0.70, p < 0.001, \text{RMSE} = 0.54) \quad (9)$$

$$\text{pH} = 2.63 + 12.50 \times \text{RIN} (R^2 = 0.67, p < 0.001, \text{RMSE} = 0.56) \quad (10)$$

At this early stage of development of 3-OH-FA based proxies for palaeoenvironmental applications, we recommend that the RAN, Branched Index and RIN should all be measured in samples, as all of them clearly have potential as pH proxies and only further work can constrain which may be most reliable or practicable.

3.3 Temperature impact on 3-OH-FAs and potential proxies

In addition to the novel pH proxies described above, we found two indices that have potential as novel temperature proxies, the ratio of *anteiso* to *normal* C₁₅ 3-OH-FA (RAN₁₅) and the ratio of *anteiso* to *normal* C₁₇ 3-OH-FA (RAN₁₇). RAN₁₅ and RAN₁₇ are defined as follows:

$$\text{RAN}_{15} = \alpha\text{-C}_{15} / n\text{-C}_{15} \text{ 3-OH-FA} \quad (11)$$

$$\text{RAN}_{17} = \alpha\text{-C}_{17} / n\text{-C}_{17} \text{ 3-OH-FA} \quad (12)$$

RAN₁₅ shows a linear relationship with MAAT and MAP ($R^2 = 0.51$ and 0.50 , respectively) (Fig. 8a, b). A similar result was also found in RAN₁₇ ($R^2 = 0.48$ and 0.48 , respectively) (Fig. 8c, d). It is not surprising that both MAAT and MAP show a linear relationship with RAN₁₅ and RAN₁₇, because both parameters strongly co-vary with elevation on Mt. Shennongjia. It has been suggested that precipitation could be an important environmental control on soil bacterial lipids in semi-arid to arid regions. Although initially proposed as

being a function of MAAT and pH, recent work has highlighted that the GDGT based MBT/CBT-MAAT index is significantly influenced by precipitation/ soil moisture in the semi-arid western USA, where MAP is below 700-800 mm yr⁻¹ (Dirghangi et al., 2013), in the semi-arid Iberian Peninsula (Menges et al., 2014) and in China (Yang et al., 2014). Yang et al. (2014) found complexities in the relationship of the MBT and CBT indices to MAAT in alkaline and arid soils in China, in contrast to their positive correlation in more acidic soils in the complete Chinese, or global, datasets. Our research area is characterised by relatively acidic to neutral soils (pH 4.5 - 8.0), and a moist-humid climate, where MAP is above 1000 mm yr⁻¹, even on the drier, lower slopes of the mountain. Therefore, we suggest precipitation/soil moisture is unlikely to be an ecologically limiting factor that significantly affects the distribution of the membrane lipids. In support of this assumption we found that both RAN₁₅ and RAN₁₇ showed very weak correlations with soil humidity measurements ($R^2=0.19$ and 0.16 , respectively, see Supplementary data Table 3), although we note that such measurements only represent the conditions at the time of sampling and not necessarily the average, mean annual conditions. Furthermore, RAN₁₅ and RAN₁₇ show significant correlations with the GDGT-based MBT/CBT-MAAT proxy published by Yang et al. (2015) on the same soil samples ($R^2=0.61$ and 0.36 , respectively) (Fig. 9a, b). Thus we assume that MAAT is the dominant parameter that affects these ratios even though the impact of MAP could not be entirely excluded. The ratios of both RAN₁₅ and RAN₁₇ increase

with decreasing environmental temperature (Fig. 8a, c). It has been observed that *anteiso* fatty acids have a lower melting point than *normal* fatty acids (Kaneda, 1991; Suutari and Laakso, 1994). Thus in order to maintain membrane fluidity, bacteria may increase the proportion of *anteiso* 3-OH-FAs (increasing the RAN indices) with decreasing temperature. This hypothesis is supported by the fact that we found a significant relationship between ratio of *anteiso* to *normal* C₁₅ 3-OH-FA and temperature, but a much less significant relationship between *iso* to *normal* C₁₅ 3-OH-FA (see Supplementary data Table 3). *Anteiso*-branched fatty acids have greater fluidizing properties and disturb packing order to a greater extent than *iso*-branched fatty acids (Russell, 1995). This is conferred by the *anteiso*-methyl branch being located on the third carbon from the methyl terminus while the *iso*-methyl branch is positioned on the second carbon from the end of the chain (Russell, 1984).

Based on the linear correlation showed in Fig. 8, we obtain the following equations:

$$\text{RAN}_{15} = 7.60 - 0.33 \times \text{MAAT} \quad (13)$$

$$\text{MAAT} = 23.03 - 3.03 \times \text{RAN}_{15} \quad (\text{R}^2 = 0.51, \text{p} < 0.001, \text{RMSE} = 2.6 \text{ } ^\circ\text{C}) \quad (14)$$

$$\text{RAN}_{17} = 2.90 - 0.11 \times \text{MAAT} \quad (15)$$

$$\text{MAAT} = 26.36 - 9.09 \times \text{RAN}_{17} \quad (\text{R}^2 = 0.48, \text{p} < 0.001, \text{RMSE} = 2.7 \text{ } ^\circ\text{C}) \quad (16)$$

The relationships of both RAN₁₅ and RAN₁₇ (equations 13 and equation 15) to MAAT are similar (see Fig. 8), although RAN₁₇ has somewhat more scatter.

GDGT data have been previously published from 19 of our 26 soil samples (Yang et al., 2015). Thus, we can directly compare our 3-OH-FA based proxies with established GDGT based proxies (CBT and MBT/CBT). Our RIAN and Branched Index proxies for pH show high linear correlation with the GDGT-based CBT (Fig. 9c, d) suggesting all three proxies have the same dominant control, namely pH. Furthermore RAN₁₅ and RAN₁₇ based on 3-OH-FA show a linear correlation with the GDGT-based MBT/CBT-MAAT proxy (Fig. 9a, b) although this is significantly higher for RAN₁₅. It is important to note that, unlike the current MBT/CBT-MAAT proxy, our proposed 3-OH-FA derived temperature proxies are independent from pH.

In addition to the ratios, indices and proposed novel proxies presented above we explored a full range of 3-OH-FA distributions (e.g. Average Chain Length of 3-OH-FAs) versus environmental parameters in the samples obtained from Mt. Shennongjia. Above we present only the most significant correlations and findings, but include all results in the Supplementary Data, Table 3.

4. Wide occurrence of 3-OH-FAs in other settings

We undertook an initial investigation to confirm the preservation of 3-OH-FAs on Quaternary time scales in several palaeoclimatic archives: a lake sediment sample dated to 1984±30 yr B.P. from Tianchi Lake, Gansu province, China, a speleothem sample dated to 8645±78 yr B.P. from Heshang Cave, China and a last glacial marine sediment sample from the 81 mbsf from IODP

Site M0060, Baltic Sea. The distribution of 3-OH-FAs varied between samples, but the suite of C₁₀ to C₁₈ *normal*, plus certain *iso*- and *anteiso*- 3-OH-FAs homologues, were all present in measurable concentrations (Fig. 10). Notably, monounsaturated 3-OH-FAs with even carbon numbers (C₁₂, C₁₄, C₁₆, C₁₈) were uniquely found in the Tianchi Lake sediment, suggesting either: a) a unique source of 3-OH-FAs in that lake environment or; b) greater preservation of the more labile unsaturated homologues (Fig. 10, Supplementary data Table 4).

The variations in the 3-OH-FA signatures between the different settings are likely due to controls by environmental and climatic parameters on membrane lipid production by bacteria (as suggested for the altitudinal transect of modern soils in this paper). Moreover, the origin and preservational pathways of 3-OH-FAs in some settings could be complex. For example, 3-OH-FAs in lake sediments may be produced *in situ* and/or may be derived from the surrounding soils, this may complicate the application of 3-OH-FAs as temperature/pH proxies in lakes. In general, we can not discount the influence on the 3-OH-FA signatures of unknown, site-specific, factors related to the differences in depositional setting or variations in populations of the Gram-negative bacterial producer. Thus specific calibrations are likely required for applications to a diverse range of palaeoclimatic archives. However, the preservation of the same suite of 3-OH-FAs in such different depositional environments, hints at a potentially wide applicability of these microbial proxies in a variety of environmental settings.

5. Conclusion

In summary, 3-OH-FAs in surface soils collected from an altitudinal transect on Mt. Shennongjia were examined to explore their relationships with environmental parameters. The RIAN, Branched index and RIN indices are highly correlated with soil pH. Furthermore, the RAN_{15} and RAN_{17} ratios exhibit significant correlations with MAAT and MAP. As precipitation is not likely to be an ecologically limiting factor in the moist-humid environment of Mt. Shennongjia we assume that MAAT is the dominant control. Notably, the 3-OH-FA based temperature proxies RAN_{15} and RAN_{17} , are not pH dependent, which should be an advantage in environments where pH is highly variable and could be a confounding variable. Our discovery of new independent proxies for pH and MAAT from an altitudinal transect of surface soils from Mt. Shennongjia has potentially wide implications for palaeoclimatic and environmental studies. 3-OH-FA proxies could be used in a variety of environmental settings (See Fig. 10). Multi-proxy terrestrial reconstructions of pH and temperature could be established by comparing 3-OH-FAs with GDGT based proxies. Gram-negative bacteria have a wide distribution in natural environment (Gupta, 1998), and 3-OH-FAs have been identified in diverse environments, including marine and terrestrial settings and even in atmospheric aerosols (Wakeham et al., 2003; Lee et al., 2004; Huang et al., 2008). In particular, these compounds are easy to identify and precisely quantify using GC-MS and GC-FID systems. This makes it possible to utilize a

small amount of sample weight and to gain high-resolution palaeo-records, for example even from stalagmite archives (Blyth et al., 2006; Huang et al., 2008; Wang et al., 2012). Additionally, measurement of 3-OH-FAs requires only standard GC-MS and GC-FID systems and can be readily adopted by most organic geochemistry laboratories (without the need for investment in additional, expensive equipment). It is clear that 3-OH-FAs have hitherto unrealized potential as palaeoclimate proxies. We hope this paper opens up new avenues of research on 3-OH-FAs, including culture studies, empirical calibrations (both global and regional) and application to an array of palaeoclimatic archives (e.g. lakes, speleothems, marine records).

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Figure captions

Fig. 1 General structure of lipopolysaccharide (LPS) from Gram-negative bacteria (Alexander and Rietschel, 2001). LPS is characterized by three main units: the O-polysaccharides chains, the core oligosaccharide and lipid A. The repeating subunits of the O-polysaccharides are composed of between one and eight glycosyl residues and differ between strains by virtue of differing sugars, sequence, chemical linkage, substitution and the ring forms utilised. The outer core is inclined to contain common sugars such as hexoses or hexosamines etc. The inner core contains the unusual sugars 3-deoxy-D-manno-octulosonic acid (Kdo) and D-glycero-D-manno-heptose (Hep) (Erridge et al., 2002). Lipid A, the innermost part of LPS, consists of two glucosamine (GlcN) moieties, with attached acyl chains ("fatty acids") by either amide bonds or ester bonds, and normally contains one phosphate group on each GlcN (Raetz et al., 2009).

Fig. 2 Regional map, illustrating the location of Shennongjia Mountain, Heshang Cave and Tianchi Lake.

Fig. 3 Cross plots showing the relationship of soil pH in samples from Mt. Shennongjia with soil humidity, Mean Annual Air Temperature (MAAT), Mean Annual Precipitation (MAP) and altitude.

751

752 **Fig. 4** Mass spectrum of the C₁₆ 3-OH-FA TMSi ester. The m/z 175 fragment is
753 due to the cleavage between C₃ and C₄, and the [M-15] base peak results from a
754 loss of a CH₃ group.

755

756 **Fig. 5** Extracted ion chromatograph (m/z 175) showing the composition and
757 distribution of 3-OH-FAs in the Mt. Shenongjia soil sample collected at 832
758 m.a.s.l. (see sample SNJ 11-4 in the Supplementary data Table 1 for more
759 detailed information). Red circles represent the *normal* 3-OH-FAs, yellow
760 squares represent the *iso* 3-OH-FAs, grey triangles represent the *anteiso* 3-OH-
761 FAs. The carbon numbers range from C₁₀ to C₁₈, including *iso* C₁₁, C₁₃, C₁₄, C₁₅,
762 C₁₆ and *anteiso* C₁₁, C₁₃ C₁₅ C₁₇.

763

764 **Fig. 6** The relationship between 3-OH-FAs indices and pH. (a) Exponential
765 correlation between the Branching Ratio and pH (R²= 0.76, p<0.001). (b) Linear
766 correlation between RIAN and soil pH (R²=0.70, p<0.001). (c) Linear correlation
767 between Branched Index and pH (R²= 0.70, p<0.001). (d) Linear correlation
768 between RIN and pH (R²= 0.67, p<0.001).

769

770 **Fig. 7** Cross plots showing the relationship between Branching Ratio and
771 Branched Index to environmental parameters (MAT, MAP, and soil humidity).

Fig. 8 The relationship between 3-OH-FA ratios and environmental factors. (a) The RAN₁₅ shows negative linear relationship with MAAT ($R^2= 0.51$, $p<0.001$) and (b) positive linear relationship with MAP ($R^2= 0.50$, $p<0.001$). (c) The RAN₁₇ shows negative linear relationship with MAAT ($R^2= 0.48$, $p<0.001$) and (d) positive linear relationship with MAP ($R^2= 0.48$, $p<0.001$).

Fig. 9 Cross plots showing the correlation between certain 3-OH-FA based and GDGT based proxies.

Fig. 10 Extracted ion chromatogram (m/z 175) showing the distribution of 3-OH-FAs in contrasting geological samples. Red circles represent the *normal* 3-OH-FAs, yellow squares represent the *iso* 3-OH-FAs, grey triangles represent the *anteiso* 3-OH-FAs and white circles represent the monounsaturated 3-OH-FAs. (a) The composition and distribution of 3-OH-FAs in a sediment sample from Tianchi Lake. (b) The distribution of 3-OH-FAs in a Heshang Cave stalagmite sample. (c) The distribution of 3-OH-FAs in Baltic Sea sediment sample.